EFFECTS OF RADIATION ON THE NUCLEOPROTEIN METABOLISM OF RABBIT LEUCOCYTES AS DETERMINED BY THE UPTAKE OF RADIOACTIVE PHOSPHORUS

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EFFECTS OF RADIATION ON THE NUCLEOPROTEIN METABOLISM OF RABBIT LEUCOCYTES AS DETERMINED BY THE UPTAKE OF RADIOACTIVE PHOSPHORUS

A Thesis
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INTRODUCTION

The research reported herein was undertaken as a culmination of training received in the radiological defense training program conducted jointly by the United States Army and United States Navy. The topic investigated was selected primarily because it offered opportunities for practical application of the principals and techniques studied in the fields of physics and the biological sciences; in addition, it was hoped that positive information would be obtained which could be useful in increasing the realm of knowledge of metabolic processes, particularly as affected by ionizing radiation.

Undue optimism was exercised in planning the scope and objectives of this project. At the outset, this research project was established with the primary objective of determining the effects of radiation on the uptake of phosphorus in the nucleic acids of rabbit leucocytes _in vitro_. Since no report of previous phosphorus metabolism studies on this type of cell could be found in the literature, it was deemed essential that investigations should initially be undertaken to determine the normal _in vitro_ phosphorus metabolism of these cells; that is, without introducing radiation effects. Studies

of this nature have been made, and are reported herein; the project in its entirety proved to be too broad in scope to be completed in the available time, and no studies of radiation effects have yet been made. It is sincerely hoped that the experiments and results herein reported shall offer sufficient stimulation to encourage some one else to continue this research beyond the present state of development.

The cost of this research was partially borne by the United States Army, through the allocation of funds for the purchase of laboratory materials and animals.

Section I. HISTORICAL DEVELOPMENT OF THE PROBLEM.

The pathological effects of ionizing radiations on biological systems are fairly well known, and general agreement as to what these gross effects are prevails among the specialists concerned with this problem. When the mechanisms which lead to these effects are considered, however, the situation is quite different. The literature on the subject is overflowing with theories and reports of experimentation attempting to prove the pet theory of whichever investigator is doing the research. Some few theories which postulate mechanisms of action have been demonstrated to be correct in a limited sense. The target theory, which is thoroughly discussed by Lea (1), can be practically applied to the consideration of damage to micro-organisms, and is probably applicable to some of the problems of the genetic effects of radiation. The indirect effect theory, postulating the creation of activated radicals in aqueous media, has been demonstrated to be a plausible explanation for many chemical reactions. The activation of water is well reviewed by Burton (2). Neither of these theories mentioned, nor any other published up to this time, can account for the full sequence of gross biological changes which

are initiated by ionising radiation. Whether these effects are brought about by the chain action of a single series of related events, or of several relatively independant series, or of many unrelated physico-chemical phenomena, can not be clearly postulated. The attendant ignorance is not fundamental, but is logically accounted for by the general lack of understanding of the minute details of the basic life processes. Thus, the faults of an electronic computer which fails to give the right answers can not be properly diagnosed without a thorough understanding of the component parts of the machine, and their interrelationships. To continue the analogy further, such a diagnosis would normally begin with an isolation and study of the basic working units of such a machine, followed by the integration of these units into the properly functioning arrangement which constitutes the whole working mechanism.

In biological systems, the basic functioning unit is considered to be the cell, since all the characteristics of life can not be demonstrated in any subordinate organization of matter. As a consequence, an understanding of cellular organization and function is deemed essential to a complete understanding of the biological phenomena resulting from any given set of circumstances.

The nucleic acids, being important constituents of living cells, have been the object of considerable

research. A number of methods of isolation and analysis of both types of nucleic acids, ribose and desoxyribose, have appeared in the literature. Less extensive research has been undertaken to study the effects of radiation of these organic acids and their protoplasmic compounds.

Mirsky and Pollister (3) isolated pure desoxyribose nucleic acid (DNA) from trout sperm. They found the nucleoprotein complex, nucleoprotamine, to be selectively soluble in 1M NaCl solution, being insoluble in water or in saline solutions of different concentrations. The pure DNA was then obtained by dialyzation of the 1M NaCl solution of the protamine against a 1M NaCl solution. In a variation of this method, (4), they also isolated the desoxyribose nucleoprotein complex from a number of mammalian tissues. Volkin, Khym, and Cohn (5) used this method to prepare pure DNA from calf thymus. This procedure appears to be quite good as a method of isolating pure DNA, but is limited in its application because of the length of time required to complete the isolation, being a matter of some weeks.

Much shorter methods of isolation and analysis are typified by the work of Schmidt and Thannhauser (6) and of Schneider (7). The method proposed by Schmidt and Thannhauser has been used in studies of the effect of radiation on nucleic acid metabolism

by Holmes (8,9) and Hevesy (10). The nucleic acid fractions cannot be quantitatively obtained by this method in reasonably pure forms. Schneider's work offers a method of a relatively rapid assay of amounts of DNA and ribose nucleic acid (RNA) in a tissue sample, but does not provide for the quantitative separation of these fractions. Assay is effected by the colorimetric determination of desoxyribose and ribose sugars, utilizing the diphenylamine reaction of Dische and the orcinol-HCl reaction, respectively.

Holmes (8,9) reported a series of in vivo nucleic acid metabolism studies on various tissues of rats subjected to ionizing radiation. Utilizing the method of Schmidt and Thannhauser, she isolated DNA and RNA from Jensen Rat Sarcoma and from rat intestine. Tissue samples were taken from rats which had been injected with tracer doses of phosphorus-32, and had been subjected to various dosages of x-irradiation. In her non-irradiated controls of rat intestine, she found that the uptake of phosphorus in the RNA fraction to be from 2 to 3 times that in the DNA fraction; in rats irradiated with whole body doses of from 200 to 2000 roentgens, this ratio of uptake increased only slightly to about 4. The uptake of phosphorus in the DNA fraction of these samples was decreased, while that in the RNA fraction remained relatively

constant. Holmes concluded that the uptake of phosphorus by DNA represents metabolic activity, but that the uptake by RNA might be merely an exchange phenomenon.

From his studies similar to those of Holmes, Hevesy (11) concluded that irradiation inhibits the formation of DNA in both growing and mature tissues, and that this inhibitory effect is predominantly transitory, lasting for a matter of some two hours. Hevesy also completed some experiments to determine whether uptake of phosphorus might in some cases be purely a physical exchange, and concluded that no such exchange occurs between phosphate groups of organic compounds and solutions of labeled inorganic phosphate (10). This latter conclusion is strengthened by the work of Osgood, et. al. (12).

Limperos and Mosher (13, 14) found that DNA was depolymerized in aqueous solution when subjected to ionizing radiation. They also showed that a similar depolymerization could be effected by chemically produced free radicals in the solution. These workers concluded from in vivo rat studies that radiation caused a splitting off of purine bases from DNA and depolymeriation of DNA. The later effect was reported to be inhibited by thiourea.

In this project, it was planned to complete a study similar to those of Holmes and Hevesy; that is,

to study the metabolic activity of the nucleic acids, as determined by the uptake of radioactively labeled phosphate. The method of Barnum and his coworkers (15, 16) was chosen for isolation of the fractions, rather than that of Schmidt and Thannhauser. The former method is a refinement of the latter, but in addition permits the separation of RNA into more specific fractions by centrifugation. Since the work of Barnum indicates that RNA is not homogeneous in its metabolic activity throughout the cell, it was deemed desirable to differentiate the RNA fractions by source. Accordingly, the decision was made to isolate three nucleic acid fractions: nuclear DNA and RNA, and cytoplasmic RNA. While the cytoplasmic RNA itself is not metabolically homogeneous, and can be further fractionated, as was done by Barnum, such a detailed procedure was not considered to be within the scope of this investigation. In their studies of mouse liver tissue, Barnum and Huseby (16) noted that the uptake in other nuclear phosphorus compounds was from 300-1200 times as great as the uptake in DNA. They did not reach a definite conclusion as to whether the slight uptake noted in the DNA was metabolically significant. The work reported herein indicates that a significant uptake of phosphorus by DNA can be demonstrated, using the same methods of fractionation, at least in *in vitro* studies of leucocytes. The method of Barnum, et. al.,

was considered to be particularly advantageous in this study as it affords recovery of the fractions in good yield, making it relatively simple to obtain significant determinations of radioactivity while subjecting the tissues to very small tracer doses of radiophosphorus.

The colorimetric determination of phosphorus directly was selected in preference to the determination of ribose and desoxyribose. Since a dichromatic method must be utilized in the determination of ribose by the orcinol-HCl reaction, to correct for interfering substances, this method is longer and subject to more error.

Section II. EXPERIMENTAL PROCEDURE AND RESULTS

Six rabbits were used in the course of this investigation. All were young, adult females, ranging in weight from approximately 3.8 to 4.5 kilograms. Diet of the animals consisted of commercial rabbit pellets supplemented weekly with fresh carrots or lettuce. The bellies of the rabbits were kept closely shaven.

Two sources of leucocytes were utilized in this work: first, leucocyte cultures were obtained from the peritoneal cavity utilizing the procedure of Weisberger, Heinle, et. al. (17); second, pure lymphocyte cultures were obtained from the vermiform appendix of freshly killed animals.

To obtain the peritoneal leucocyte cultures, the rabbits were injected intraperitoneally with 300 milliliters of sterile physiological saline (.85% NaCl) at approximately 4:00 P.M. on one day, followed by a similar injection at 9:00 A.M. the following day. Four hours following the second injection, an exudate, rich in leucocytes, was withdrawn from the peritoneal cavity, utilizing a sterile number 18 needle attached to a dry 50 milliliter syringe. The needles utilized were modified by the addition of 3 side holes bored

near the tip to facilitate the flow of exudate and decrease the likelihood of blocking by the viscera. During all insertions, the rabbit board was tilted approximately 45 degrees, the posterior end of the animal being elevated, so that the viscera would tend to move anteriorly and out of the path of the needle. The animal was left in this position during the injections, but was returned to a normal horizontal position immediately after the insertion of the needle for withdrawal of the exudate. The time required for injection was somewhat variable, but generally short, lasting from 10 to 30 minutes. Successful recovery of exudate was usually effected in less than 30 minutes, or not at all. A recovery of at least 40 milliliters of exudate was considered successful, although about twice this much is preferable for successful analytical procedures. In the six animals used, recovery varied from 0 to 125 milliliters, generally running around 60 to 80 milliliters. The only visible effect on the rabbits of the entire procedure was a mild diarrhea following injection. Some exudative cultures were rejected due to considerable erythrocyte contamination. To prevent such contamination, several precautions were taken. The rabbits were handled as gently as possible to reduce their fright and keep them in a quiet, relaxed state during the procedure; most of the rabbits became quite

acclimated to the process, and gave very little trouble. Insertions were always made in the midline, to decrease the possibility of rupturing blood vessels. A recent paper (22) suggests a simple method of overcoming this difficulty. The exudate could be centrifuged in a light plastic container, such as a plastic straw, and then quick frozen. Any erythrocytes in the culture would be in a layer above the leucocytes, being lighter, and could be readily cut away with a razor blade, leaving a pure leucocyte culture.

Pure lymphocyte cultures were obtained from the vermiform appendix of four rabbits. To obtain such a culture, a rabbit was killed between the hours of 10:30 A.M. and 12:30 P.M., by suffocation with a chloroform mask. The appendix was removed, washed in physiological saline, and then opened down one side, so that the mucousa was completely exposed, and the tissue could be laid out flat. The epithelial lining was then stripped back from the tissue with tweezers, exposing a mass of lymphocytes, which were gently scraped loose and removed with a scalpel. The recovery of the free lymphocytes from the excised appendix required from 5 to 10 minutes. During the procedure, the tissue was kept bathed with physiological saline.

Handling of both types of cell samples was essentially identical after recovery. The samples were

washed in buffered Ringer-Evans Solution, the composition of the solution being as shown in Table I. The solution was checked prior to use on a Beckman pH Meter, and brought to pH 7.4 by the addition of 0.1N NaOH or 1N H_3PO_4 as necessary. As a rule, the pH of the solution was generally low, and the addition of a small amount of hydroxide was required. After washing, the suspensions were centrifuged and the supernate pipeted off. The washed cells were then incubated at room temperature in heparinized Ringer-Evans Solution with a tracer dose of phosphorus-32 added as H_3PO_4. Tracer doses of phosphate with activities varying from 2 to 6 microcuries of phosphorus-32 were utilized. Exudative leucocytes were incubated for 30 minutes in 15 milliliters of Ringer-Evans Solution with 5 milligrams of heparin and from 2 to 3 microcuries of phosphorus-32 added. These solutions were not checked for pH after addition of the radioactive phosphoric acid. These solutions were occasionally stirred by hand with a glass rod during the incubation period. Lymphocytes were incubated for 30 minutes in 25 milliliters of Ringer-Evans Solution, brought to pH 7.4 after the addition of 7.5 milligrams of heparin and approximately 5 microcuries of phosphorus-32. During the entire incubation, the suspensions were stirred gently with a glass rod driven by a stir motor. After 30

minutes incubation, suspensions were centrifuged for 15 minutes with a centrifugal force of 500x gravity (Setting #10 on International Equipment Company Size 1 Model 250 Centrifuge with head number 221, utilizing 40 milliter centrifuge tubes).

After recovery of the washed cells, nucleic acid fractions were recovered following the procedures of Lerman and coworkers (15, 16). The detailed steps of fractionation are outlined in Charts I, II and III. The construction and description of the Potter-Elvehjem Homogenizer used are outlined in Appendix I.

To verify successful separation of the cells into nuclear and cytoplasmic components, microscopic slides were habitually made of the washed nuclei after completion of the citric acid washes, and stained for two minutes with Wright's Stain. Under the oil immersion lens, these slides showed intact nuclei, with only occasionally some slight debris.

Three nucleic acid fractions were recovered and analyzed in this investigation, the fractions being the cytoplasmic ribose nucleic acid, the nuclear ribose nucleic acid, and the nuclear desoxyribose nucleic acid. In addition, a sample of the radioactive inorganic Ringer-Evans Solution from each incubation was saved and analyzed.

Phosphorus content of each fraction was determined by the method of LePage (18). Monobasic potassium

phosphate was used as the standard solution. Most analyses were run against a standard of 0.1 microgram of phosphorus per milliliter. Colorimetry was accomplished on an Evelyn Photoelectric Colorimeter, using the macro unit, with a 540 millimicron filter. This wavelength was found to give good results over a wide range of phosphorus concentrations. A standard curve was plotted on semi-logarithmic paper showing concentration of phosphorus as linear abscissa versus colorimeter reading as logarithmic ordinate. The best curve to fit the points plotted was determined statistically by finding the regression line of log reading on concentration, as outlined by Fisher (19). The resulting standard curve is shown as Graph I. Colorimeter tubes used were etched at the 10 milliliter level. When it was found necessary further to dilute some samples to obtain good colorimeter readings, dilutions were accomplished by adding either 10 or 20 milliliters of doubly distilled water with a 10 milliliter volumetric pipette. The colorimeter tubes were regularly cleaned with cleaning solution and rinsed with doubly distilled water prior to use. The trichloroacetic acid used in the fractionation process was tested for phosphorus contamination as recommended by Fiske and Subbarow (20), and found to be phosphorus-free. The 1, 2, 4 amino-naphthol-sulfonic acid used in preparing the Fiske and Subbarow reducing agent was recrystallized as recommended

by them prior to use. This reducing agent was kept well stoppered in a brown bottle, and in a closed cupboard, and a new stock was prepared about every four weeks. Results of the analyses made indicate that the solution probably should have been renewed more often, possibly every two weeks.

After the completion of the colorimetric analysis, the samples were evaporated down in aluminum planchets on a hot plate at a low temperature. Samples were counted to determine the phosphorus-32 activity in a proflush flow counter, using a Tracerlab Autoscaler to record the count. All fractions from the same original sample, including the inorganic supernate, were counted at the same time, so that the use of decay corrections was not required. About 0.1 milliliter of the supernate containing inorganic phosphorus was found to be a satisfactory sample for both the colorimetric determination of phosphorus, and for the determination of radioactivity. Accordingly, 10 milliliters of this supernate was diluted to 100 milliliters in a volumetric flask, and a 1 milliliter aliquot was utilized as the sample.

In analyzing the lymphocyte fractions, it was found desirable to take a 5 or 10% aliquot part of the two nuclear fractions for colorimetric analysis. After these samples were wet ashed, they were brought up to the 10 milliliter mark in the colorimeter tubes. In

aliquot part consisting of either 0.5 milliliter or 1 milliliter was then withdrawn with a serological pipette and placed in a clean colorimeter tube. The pipette in each case was then washed with distilled water, washings being added to the original tube from which the aliquot was drawn. This washing was done to prevent any loss of radiophosphorus. The appropriate amount of 10N H_2SO_4 was then added to the aliquot, so that the fraction actually analyzed contained 0.4 milliliters of acid, as required.

Results of analyses of the sensitive leucocytes and lymphocytes are tabulated in Tables II and III, respectively. Colorimeter readings listed in these tables are corrected readings; i.e., corrections to account for the non-linearity of the galvanometer scale, as furnished by the manufacturer, have been applied. Relative specific activities listed have been determined by dividing the specific activity of a nucleic acid fraction by the specific activity of the corresponding inorganic fraction.

Since the specific activity of the inorganic supernate is not constant among the various experiments, comparisons can only be made between the results of the different experiments on the basis of the relative specific activities, as defined above.

Section III. DISCUSSION OF THE RESULTS OF EXPERIMENTATION

Analyses were carried out on five samples of exudative leucocytes, and four samples of lymphocytes, from young adult rabbits, after incubation *in vitro* in buffered Ringer-Evans Solution containing tracer doses of phosphorus-32 as phosphate.

The data in Table II, obtained from the analysis of the leucocyte samples, give little insight into the relative phosphorus metabolism of the nucleic acid fractions analyzed. Analyses of the cytoplasmic ribose nucleic acid from sample 10 and of the desoxyribose nucleic acid from sample 12 were incomplete, as a partial loss of these fractions was incurred before their radioactivity was determined. In the case of the latter, the loss occurred when the fraction was being evaporated in an aluminum planchet on a hot plate. The planchet developed a leak, possibly because the hot plate was too hot, although the same difficulty did not occur in any of the other fractions being evaporated at the same time. Subsequent to this accident, all planchets were carefully inspected for faults prior to use, and care was exercised to insure that the hot plate used for evaporation of the samples was kept at a low temperature. The relatively low recovery of phosphorus

[illegible]

[illegible]

[illegible]

[illegible]

from the cytoplasmic ribose nucleic acid of samples 11 and 12 lead to activities in these samples very close to background. As a consequence, the results of analyses of these two samples are statistically not as significant as the results obtained from analyses of the other samples.

Assuming that the recovery of phosphorus in the various fractions analyzed is roughly quantitative; that about 70% of the nuclei are recovered after homogenization; and that phosphorus represents about 10% of the total weight of both ribose and desoxyribose nucleic acid, all assumptions being based on the work of Barnum, et. al. (15), the consideration of the recovery of phosphorus in all leucocyte fractions indicates that the relative amounts of desoxyribose nucleic acid, nuclear ribose nucleic acid and cytoplasmic ribose nucleic acid in these cells show a ratio on the order of 30:5:1.

The analyses of the lymphocyte samples, the results of which are listed in Table III, were more successful than the analyses of the leucocyte samples. No lymphocyte fraction was lost during the course of the analyses. The net activity of each fraction analyzed was sufficiently great as compared to background to be statistically significant. The activity of the cytoplasmic ribose nucleic acid fraction of sample 13 was quite low, but the significance of the

count was increased by utilizing a high total count. If one accepts the assumptions in the previous paragraph, the consideration of the recovery of phosphorus from all lymphocyte fractions indicates that the relative amounts of desoxyribose nucleic acid, nuclear ribose nucleic acid and cytoplasmic ribose nucleic acid in these cells show a ratio of the order of 30:2:1.

The mean values are 0.0016, 0.033 and 0.021, respectively, for the relative specific activities of the desoxyribose nucleic acid, nuclear ribose nucleic acid and cytoplasmic ribose nucleic acid from the lymphocyte samples. The standard errors of the mean for these three mean values, computed according to Treloar (20), are 0.00022, 0.001 and 0.004. Accordingly, one may state, with approximately a 68% chance of being right, that the true mean values lie within the regions 0.0008–0.0024, 0.012–0.054 and 0.013–0.029, respectively. These considerations show that, with the wide dispersion obtained in the results, little weight can be attached to the final value shown in Table III for the ratio of the mean values of the relative specific activities of the three nucleic acid fractions.

A study of the data of both Table II and Table III has been made to determine if any uncontrolled variables in the experiments can be correlated with the wide range of results obtained.

According to Hevesy (10), phosphomolybdate has a significant absorption coefficient for the 1.7 million electron volt (MEV) beta particles emitted by phosphorus-32. Since the phosphorus of the nucleic acid fractions in this investigation was incorporated into a phosphomolybdate complex, the data were examined to determine whether self-absorption in the samples might be a cause of some variations. No correlation could be found between the amounts of phosphorus recovered in the fractions and their net activity to indicate that self absorption might be a pertinent factor.

All samples analysed were incubated in radioactive Ringer-Evans Solution at room temperature. During the course of these experiments the temperature of the laboratory did not vary more than 3 degrees centigrade at most. Since the incubation periods were of 30 minutes duration only, any such variations in temperature would not likely affect the rate of uptake of phosphorus by the cells to any significant degree.

The radioactive solutions in which the exudative leucocytes were incubated were not checked for pH subsequent to the addition of the radioactive H_3PO_4. Ringer solutions in which the lymphocytes were incubated were so checked, however, and the pH corrected to 7.4 where necessary. As a consequence, pH of

the suspensions might be a factor causing some variation in the results of analysis of the leucocyte samples, but not of the lymphocyte samples. Since the Ringer-Evans Solution is buffered, and the number of equivalents of H_3PO_4 added in any case was quite small, the probability is small that large pH changes occurred in the leucocyte suspensions following the addition of the H_3PO_4.

There is a possibility that the inorganic fractions from the radioactive Ringer-Evans Solution were in some cases contaminated with organic phosphorus. Such contamination might account for the variation in specific activity among the inorganic fractions of samples 16, 17 and 18, each of which included the same amount of radioactive phosphorus. Errors in the determination of the specific activities of the inorganic fractions, caused by such contamination, would tend to invalidate the comparisons between relative specific activities of the nucleic acid fractions among the several samples. Such errors would not be random in nature, since contamination of the inorganic phosphorus would always lead to too low a result in the determination of the specific activity in an inorganic fraction. Since the inorganic phosphorus does not need to be wet ashed prior to the colorimetric determination of phosphorus as orthophosphate, variations due to contamination by organic

phosphorus could be largely eliminated by omitting the wet ashing of the inorganic fractions prior to the colorimetric determination of phosphorus. If the wet ashing were eliminated, contamination of an inorganic fraction by organic phosphorus would tend to cause a slight increase in the specific activity of the fraction, as the radioactivity of the contaminant would cause an increase in the measured net activity. Such an increase would be small, however, since, under the conditions of the investigation, the specific activity of the radioactive Ringer-Locke Solution has generally proved to be considerably greater than that of any of the organic fractions.

Section IV. CONCLUSIONS

Based on the data presented herein, the following conclusions have been reached:

(1) The techniques utilized in the isolation and analysis of the nucleic acid fractions of rabbit leucocytes and lymphocytes are suitable for determining the relative metabolic uptake of phosphorus under the conditions of the experiments.

(2) Insufficient data have been accumulated to permit the computation of a statistically significant ratio indicating the relative uptake of phosphorus among the fractions and under the conditions specified. The data do indicate that, in the lymphocytes, uptake of phosphorus is most rapid in the nuclear ribose nucleic acid and least rapid in the desoxyribose nucleic acid; in the leucocytes, uptake is least rapid in the desoxyribose nucleic acid.

(3) In both types of cultures studied, the preponderance of nucleic acid phosphorus is contained in the desoxyribose fraction.

(4) With the accumulation of further data, an extension of the experiments to show the effects of radiation on the relative uptake of phosphorus among the nucleic acid fractions studied should be feasible.

Compound	Amount, grams
H_2O	100
NaCl	0.85
KCl	0.042
$CaCl_2$	0.024
Glucose	0.1
Na_2HPO_4 or $Na_2HPO_4 \cdot 12H_2O$	0.06 0.151

Adjust to pH 7.4 with 1N H_3PO_4 or 0.1N NaOH.

Table I. Composition of Buffered Ringer-[illegible] Solution (21).

Rabbit Number	Quantity of Exudate, ml.	Sample Number	Tracer Dose, μc P^{32}	Fraction	Colorimeter Reading	Total Phosphorus, μg	Count/Counting Time, (sec)	Background, counts per second	Net Activity, counts per second	Specific Activity, cps/μg P	Relative Specific Activity	Ratio of Specific Activities, ND:NR:CR	Remarks
851 861	60 10	8	2.51	ND8	17^1	204	512/67.0	0.80	7.59	0.037	0.012	1:14:14	(a), (b)
				NR8	46^2	22.5	1024/75.2		12.8	0.54	0.17		
				CR8	66^3	12.8	1024/133		6.89	0.54	0.17		
				IN8	93^1	3.0	512/51.0		7.24	3.1		—	0.02 ml. sample
860	60	9	2.00	ND9	24^2	85.0	1024/110	0.80	8.51	0.10	[illegible]	1:[illegible]	(a)
				NR9	14^2	62.5	1024/73.2		13.2	0.21	[illegible]		
				CR9	64	14.1	1024/166		5.37	0.38	[illegible]		
				IN9	82^2	6.6	1024/127		15.0	14			
433	50	10	3.00	ND10	45^2	48.6	1024/93.1	0.80	11.0	0.31	0.016	1:[illegible]	(a)
				NR10	38^2	21.5	1024/43.0		22.0	0.78	[illegible]		
				CR10	10^1	3.9	—		—	—	—		(d)
				IN10	87^1	4.2	1024/9.3		107	[illegible]			
857	70	11	2.14	ND11	41	221	2048/156	1.01	12.1	0.055	[illegible]	1:18:[illegible]	(c)
				NR11	60^1	15.8	2048/122		15.8	1.0	[illegible]		
				CR11	85^2	5.1	2048/727		1.81	[illegible]	[illegible]		
				IN11	86^1	5.3	2048/22.9		88.5	17			
860	85	12	2.56	ND12	45^2	242	—	0.75	—	—	—	[illegible]	(c), (d)
				NR12	15	61.2	2048/262		6.88	0.11	[illegible]		
				CR12	75	9.5	2048/666		2.13	0.22	[illegible]		
				IN12	87^3	7.6	4096/30.2		112	15			

Remarks:
(a) Colorimeter sample made to 20 ml.
(b) 5 μg P used as colorimeter standard.
(c) 0.1 aliquot used for colorimeter sample.
(d) Sample lost prior to counting.

ND - Desoxyribose Nucleic Acid
NR - Ribose Nucleic Acid from Nuclei
CR - Ribose Nucleic Acid from Cytoplasm
IN - Inorganic Supernate

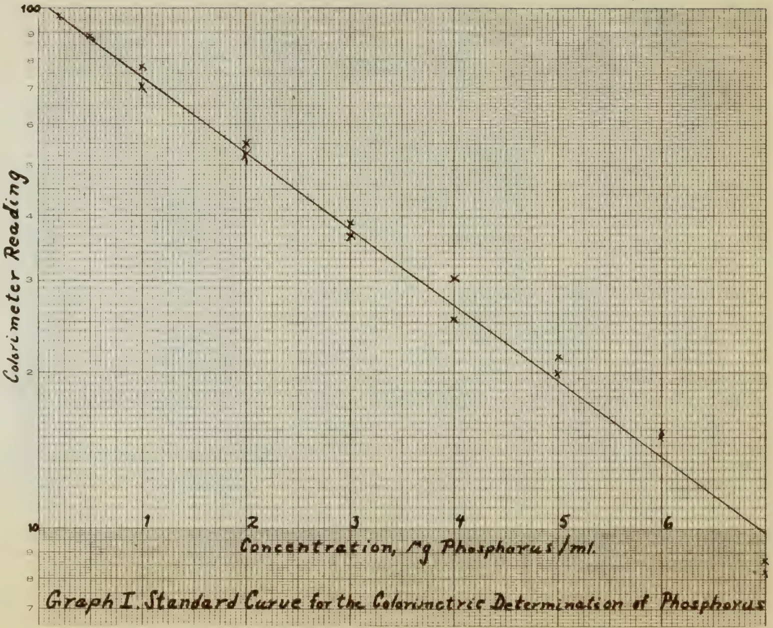
Table II. Results of Analysis of Leucocyte Samples.

[illegible]	Hematocrit (c)	Sample Number	Tracer Dose, μc P^{32}	Fraction	Colorimeter Reading	Total Phosphorus μg	Counts/Counting Time, (sec)	Background counts per second	Net Activity Counts per second	Specific Activity cps/μg P	Relative Specific Activity	Ratio of Specific Activities, ND:NR:CR	Remarks
861	2.5	15	5.02	ND15	12^2	625	2048/345	0.92	5.01	.0080	.00050	1:19:8.2	(c)
				NR15	17^3	52.0	2048/250		7.97	.15	.0094		
				CR15	42^2	26.0	8192/5078		1.71	.065	.0041		
				IN15	30^2	7.4	8192/46.9		117	16			
860	2.5	16	5.13	ND16	67^3	1540	4096/244	1.08	15.7	.010	.00056	1:25:21	(b) (c) (f)
				NR16	25^1	83.6	4096/179		19.5	.23	.013		(2)
				CR16	11	66.2	4096/277		13.7	.21	.012		
				IN16	[illegible]	11.8	4096/19.1		213	18			
433	2.5	17	5.13	ND17	23^2	868	4096/104	1.03	38.5	.044	.0040	1:23:11	(d)
				NR17	86^1	54	4096/71.0		56.7	1.0	.091		(c)
				CR17	17^1	53.0	4096/154		25.6	.48	.044		
				IN17	84^2	5.7	4096/44.1		94.9	11			
432	3.0	18	5.13	ND18	27^1	1470	2048/65.5	1.24	31.1	.021	.0015	1:13:16	(2) (a)
				NR18	79^2	71	2048/57.1		21.6	.28	.020		(c)
				CR18	39^2	51	2048/99.6		14.5	.34	.024		(c)
				IN18	75^1	5.1	2048/44.8		44.4	14			
Mean Values									ND		.0016	1:21:13	
									NR		.033		
									CR		.021		

Remarks:
(2) Colorimeter sample made to 25 ml.
(a) " " " " 30 ".
(c) 0.1 aliquot used for colorimeter sample.
(d) 0.05 " " " " " .
(e) Ml. of packed cells after 15 min. centrifugation at 500 x gravity.

(1, 1ⁿ, ...) used as colorimeter standard.
ND - Desoxyribose nucleic acid.
NR - Ribose nucleic acid from nuclei.
CR - " " " " cytoplasm.
IN - Inorganic supernate.

Table III. Results of Analyses of Lymphocyte Samples.



Graph I. Standard Curve for the Colorimetric Determination of Phosphorus

Homogenize in 4 volumes ice cold saline (0.85% NaCl containing 2 ml 0.1N NaOH/liter) 2 minutes. The Potter-Elvehjem tube and pestle should be ice cold.

Centrifuge (1400xgravity^a, 4 minutes)

Supernate 1

Residue 1
Resuspend in cold 2%
Citric Acid.

Centrifuge (500xgravity^b, 10 minutes)

Supernate 2
Add to
Supernate 1

Residue 2
Repeat 2 times for 5 min. ea.
" 5 " " 3 " "
" 3 " " 1 " "

Supernates 3-12
Add to
Supernate 1

After last 1 minute spin,
stir and respin to avoid
loss of nuclei.

Cytoplasmic
Extract

Remarks: a. Centrifuge setting #20 (on International Equipment Co., Size 1, Model #50 centrifuge with head number 213), using 40 milliliter centrifuge tubes).
b. Centrifuge setting #10.

Chart I. Homogenization of Cells and Separation into Cytoplasmic and Nuclear Fractions. (After Barnum, et.al.) (15).

Make the cytoplasmic extract up to 10% with NaCl as soon as it is obtained from the centrifugal fractionation. Heat in a boiling water bath 30 minutes.

Centrifuge

Supernate 1
Precipitate for two hours after adding 2 volumes of absolute ethanol.

Residue 1
Wash with hot 10% NaCl and add washings to Supernate 1. Discard residue.

Centrifuge

Supernate 2
Discard

Residue 2
Dissolve in water. Add cold TCA* to obtain a final concentration of 5%.

Centrifuge

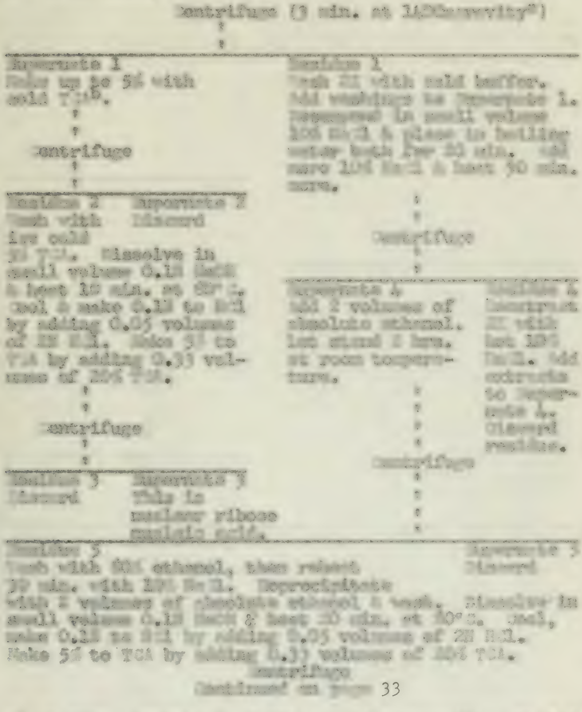
Supernate 3
Discard

Residue 3
Wash with cold 5% TCA, then hydrolyze in 5% TCA at 90-95° C for 30 minutes. The product is relatively pure ribose nucleic acid.

*Trichloroacetic Acid

Chart II. Isolation of Ribose Nucleic Acid From the Cytoplasmic Extract. (After Barnum and Huseby) (16)

After the citric acid washes, wash nuclei 3x with 0.85% NaCl. Extract with 95% ethanol. Extract 3x with boiling absolute ethanol-ether (3/1). Extract with 95% ethanol. After each washing or extraction centrifuge and discard the supernate. Resuspend the residue in ice cold buffer at pH 10 (equal volumes of 0.1M Na_2CO_3 and 0.1M $NaHCO_3$). Stand 30 minutes in ice water bath.



Centrifuge (3 min. at 1400×gravity*)

Supernate 1
Make up to 5% with cold TCA.

Centrifuge

Residue 2
Wash with ice cold 5% TCA. Dissolve in small volume 0.1N NaOH & heat 10 min. at 80°C. Cool & make 0.1N to HCl by adding 0.05 volumes of 2N HCl. Make 5% to TCA by adding 0.33 volumes of 20% TCA.

Supernate 2
Discard

Centrifuge

Residue 3
Discard

Supernate 3
This is nuclear ribose nucleic acid.

Residue 1
Wash 2x with cold buffer. Add washings to Supernate 1. Resuspend in small volume 10% NaCl & place in boiling water bath for 30 min. Add more 10% NaCl & heat 30 min. more.

Centrifuge

Supernate 4
Add 2 volumes of absolute ethanol. Let stand 2 hrs. at room temperature.

Residue 4
Reextract 2x with hot 10% NaCl. Add extracts to Supernate 4. Discard residue.

Centrifuge

Residue 5
Wash with 80% ethanol, then reheat 30 min. with 10% NaCl. Reprecipitate with 2 volumes of absolute ethanol & wash. Dissolve in small volume 0.1N NaOH & heat 10 min. at 80°C. Cool, make 0.1N to HCl by adding 0.05 volumes of 2N HCl. Make 5% to TCA by adding 0.33 volumes of 20% TCA.

Supernate 5
Discard

Centrifuge

Continued on page 33

Chart III. Isolation of Ribose and Desoxyribose Nucleic Acids from Frog Nuclei. (After [illegible], et. al.) (13).

Residue 6 Wash with 5% TCA & hydrolyze with 5% TCA by heating 30 min. at 90°C. This final solution is desoxyribose nucleic acid.	Supernate 6 Discard

Remarks: (a) Centrifuge setting # 20 (on International Equipment Co., Size 1, Model 690 Centrifuge with head number 221), using 40 milliliter centrifuge tubes).

(b) Trichloroacetic acid.

Appendix I. CONSTRUCTION AND DESCRIPTION OF POTTER-ELVEHJEM HOMOGENIZER

The Potter-Elvehjem homogenizer used in this work was constructed from a 20x150 millimeter Pyrex test tube. The inside of the tube was ground slightly with a light suspension of carborundum powder in water. A smaller test tube fitted with a one-hole stopper and a glass stirring rod was used as a pestle, and driven on a stirring motor, to effect the grinding.

The pestle for the homogenizer was made from a piece of Lucite rod fitted with a 3/16 inch brass rod 19 centimeters in length. The Lucite rod, 2.5 centimeters long, was center drilled on a lathe to a depth of 1.5 centimeters and the brass rod was set in the Lucite with a thick mixture of plastic resin glue. The Lucite head was then ground with fine emory paper until it fitted the tube. The fit was considered proper when the tube, filled with water, would slowly drop off the pestle. Potter (16) states that this represents a clearance between tube and pestle of approximately 0.1 millimeters. The lower edge of the Lucite was then beveled to fit the contour of the curved bottom of the tube, and the tip was scored along several diameters with a coping saw. The latter step was taken in lieu of placing protruding beads on the bottom, as recommended by Potter. Beads were

considered unnecessary since the homogenizer was to be used only on free cells, and not on pieces of whole tissue which would require tearing and shredding.

The homogenizer pestle was driven by an air-driven stir motor. In use, just enough force was supplied to the motor so that friction would not stop the pestle.

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14. [illegible]

15. [illegible]

16. [illegible]

17. [illegible]

18. [illegible]

19. [illegible]

20. [illegible]

21. [illegible]

22. [illegible]

23. [illegible]

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